乳腺特异性表达催乳素基因载体的构建与检测

任艳萍[1,2] 雷小灿[1,2] 谢亮亮[1] 罗 婵[1] 刘晓华[1] 石德顺[1]* 李湘萍[1]*

([1]广西大学, 亚热带农业生物资源保护与利用国家重点实验室, 南宁 530004; [2]遵义医学院基础医学院, 遵义 563003)

摘要 催乳素(prolactin, PRL)可通过PRL-PRLR-JAK/STAT信号通路促进乳腺发育, 启动并维持泌乳。为了探讨调控*PRL*基因表达对奶水牛产奶量的影响, 该研究构建了乳腺特异性表达*PRL*基因的核移植载体并检测了其有效性。首先, 利用RT-PCR方法克隆得到804 bp的水牛*PRL*基因编码区; 而后逐步采用酶切加连接方法, 依次将*PRL*基因、β-酪蛋白(β-casein, *BCN*)启动子和标记基因插入pIFN-BCNpolyA质粒中, 构建得到14.2 Kb的转*PRL*基因载体。将表达载体瞬时转染人Bcap-37细胞系, 经RT-PCR检测发现, 目的基因*PRL*可在该细胞系中表达。将该载体转入水牛胎儿成纤维细胞中, 通过核移植法获得了转*PRL*基因水牛克隆胚胎。该文结果表明, 所构建的*PRL*核移植载体可表达*PRL*基因, 并可用于生产转*PRL*基因克隆水牛胚胎。

关键词 PRL; 乳腺特异性表达; 载体构建; 转基因动物; 核移植

The Construction and Detection of Nuclear Transfer Vector Specifically Expressing *PRL* Gene in the Mammary Gland

Ren Yanping[1,2], Lei Xiaocan[1,2], Xie Liangliang[1], Luo Chan[1], Liu Xiaohua[1], Shi Deshun[1]*, Li Xiangping[1]*

([1]*State Key Laboratory of Subtropical Bioresource Conservation and Utilization, Guangxi University, Nanning 530005, China*; [2]*School of Basic Medical Sciences, Zunyi Medical University, Zunyi 563003, China*)

Abstract Prolactin (PRL) could improve the development of mammary gland, promote and maintain the lactation by PRL-PRLR-JAK/STAT signaling pathway. In order to investigate the influence of the buffalo mike yield regulated by *PRL* gene expression, the nuclear transfer vector specifically expressing *PRL* gene in the mammary gland was constructed, and its effect was detected in this study. At first, the 804 bp *PRL* gene coding region of female buffalo was cloned by RT-PCR method. The 14.2 Kb PRL transgenic vector was constructed by inserting *PRL* gene, *BCN* (β-casein) promoter and marker gene into pIFN-BCNpolyA plasmid using restriction and ligation methods. The PRL vector was transiently transfected into human Bcap-37 cell line, and the expression of *PRL* target gene was detectable in the transfected cells. Finally, the vector was transfected into the buffalo fetal fibroblast cells, and the *PRL* transgenic cloning buffalo embryos were produced by nuclear transfer method. Our results indicated that the constructed vector could express *PRL* gene, and could be applied for producing *PRL* transgenic cloning buffalo embryos.

Keywords PRL; the mammary gland special expression; the vector construction; transgenic animal; nuclear transfer

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*通讯作者。Tel: 0771-3239202, E-mail: ardsshi@gxu.edu.cn; Tel: 0771-3239202, E-mail: xiangpingli@163.com

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*Corresponding authors. Tel: +86-771-3239202, E-mail: ardsshi@gxu.edu.cn; Tel: +86-771-3239202, E-mail: xiangpingli@163.com

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牛奶是人体钙与蛋白质的极佳来源, 加之其所含氨基酸种类全面, 微量元素丰富, 有着很高的营养价值。水牛在我国南方存栏量大, 奶品质优良, 但本地奶水牛奶产量低, 急需采用多种方法培育品质优良、奶产量高的新型奶水牛。与传统育种手段相比, 分子育种的周期明显缩短, 尤其是结合体细胞核移植技术, 更可在短时间内培育出遗传性状稳定的新型奶水牛。

腺垂体分泌的催乳素(prolactin, PRL)因其具有促进乳腺发育、启动并维持泌乳的作用, 而被命名为催乳素[1]。研究发现, 其主要通过PRL-PRLR-JAK/STAT信号通路发挥泌乳功能。PRL首先与PRL受体(prolactin receptor, PRLR)识别并结合[2-3], 迅速诱导PRLR同源二聚化[4-6], 激活与之相邻的JAK2(Janus tyrosine kinase 2), 使其发生自身酪氨酸磷酸化[7-8], 多数情况下还会伴随发生PRLR的磷酸化[9-10]。活化JAK2酪氨酸磷酸化Stat5(signal transducers and activators of transcription 5)[11-12], 2个被磷酸化的Stat5形成二聚体, 转移到细胞核中, 识别并激活特异性的应答原件, 调控靶基因的表达[13-15], 从而发挥其功能。敲除*PRL*基因后小鼠乳腺发育出现障碍, 无法分化出正常腺泡细胞, 也无法泌乳[16]。敲除PRL信号通路末端的*Stat5*基因, 会出现乳腺发育障碍、泌乳量下降或无法泌乳的现象[17-18]。单独使用PRL激素处理假孕兔, 即可引发假孕兔乳腺腺泡分化, 腺泡腔内出现大量的脂肪球和蛋白质胶粒[19]。以上研究表明, PRL在促进乳腺发育、启动并维持泌乳方面具有重要作用。

适量的PRL表达可以促进乳腺发育以及乳汁分泌, 但全身范围的PRL过量表达会增加乳腺肿瘤的发生机率, 如全身过表达PRL转基因小鼠的乳腺肿瘤发病率显著上升[20], 绝经后妇女血清中PRL的含量与乳腺癌发生机率正相关[21]。因此, 在利用外源表达*PRL*基因促进乳汁分泌的同时, 需要限制外源*PRL*基因的表达区域。为此, 本文选择了乳腺特异性表达启动子以限制*PRL*的表达范围。前期实验中, 我们采用不含筛选基因*NEO*的转*PRL*基因载体, 通过受精卵胞质注射法生产了转基因小鼠。在雌泌乳期性转基因小鼠的乳腺组织中检测到外源PRL蛋白质表达, 表达的PRL具有生物活性, 并可显著提高转基因小鼠乳腺中泌乳相关基因β-1,4-半乳糖转移酶1和β-酪蛋白的表达水平。除小鼠外, 受精卵胞质注射法生产转基因动物存在效率低的问题, 不适用于繁殖能力低、繁殖周期长、成本高的水牛, 为此本文构建了适用于核移植方法的*PRL*转基因载体, 并检测了该载体的有效性。

1 材料与方法

1.1 材料

垂体组织样本采自广西南宁鲁班路屠宰场的成年雌性水牛, 垂体组织分装到DEPC处理过的EP管中, 液氮保存。

1.2 *PRL*基因的克隆

根据NCBI上牛*PRL*的mRNA序列(NM_173953.2)设计特异性引物。用Trizol法提取成年雌性水牛垂体总RNA, 并反转录为cDNA, 以其为模板, SiPRL-F/PRL-R为引物(表1), 扩增*SiPRL*片段。反应条件为: 94 ℃预变性4 min; 94 ℃变性30 s, 60.5 ℃退火30 s, 72 ℃延伸55 s, 重复35个循环; 72 ℃再延伸6 min; 12 ℃保存。胶回收后, TA克隆到pMD18-T载体中, 得到pPRL质粒。以该质粒为模板, PRL-F/PRL-R为引物(表1), 扩增PRL片段, PCR反应条件同上。胶回收目的片段后, TA克隆得到pSiPRL质粒, 并将该质粒送到生工上海生物工程有限公司进行测序。

1.3 乳腺特异表达载体的构建

乳腺特异表达载体的构建, 是以在*Kpn* I/*Sac* I位点插入有BCNpolyA序列的pMD18-T质粒(pIFN-BCNpolyA)为骨架, 依次插入*PRL*基因、乳腺特异性*BCN*(β-casein)启动子(娟姗牛β-酪蛋白启动子)、标记基因3个片段。具体步骤为: (1)利用*Kpn* I/*Bam*H I双酶切、T4连接, 插入5′-*Bam*H I-PRL-*Knp* I-3′片段; (2)利用*Bam*H I/*Sal* I双酶切、T4连接, 插入5′-*Sal* I-*Not* I-5.2BCN-*Bam*H I-3′片段; (3)利用*Sal* I/*Not* I双酶切、T4连接, 插入5′-*Sal* I-标记基因-*Not* I-3′片段, 其中标记基因为cmv-EGFP-IRES-neo-SV40polyA。最终构建得到p5.2BCN-PRL-BCNpolyA-cmv-EGFP-IRES-neo-SV40polyA这个乳腺特异性表达水牛*PRL*基因的核移植载体。将该载体分别用*Bam*H I/*Kpn* I和*Not* I/*Sal* I进行酶切鉴定。

1.4 Bcap-37细胞转染与检测

采用无内毒素质粒试剂盒提取转基因载体, 并

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用无内毒素TE buffer将载体浓度调整至1 μg/μL备用。培养Bcap-37细胞系, 待汇合度达90%时, 用脂质体Lipofectamine® LTX with Plus™ Reagent分别转染转基因载体和TE buffer阴性对照。转染24 h后观察荧光; 48 h后收集细胞。用Trizol法提取细胞RNA, DNase I处理后, 反转录得到cDNA, 并用*Dpn* I处理, 去除残留的质粒DNA。处理过的细胞cDNA为模板, 未经反转录的细胞RNA为阴性对照, 水为空白对照, 以PRL-F/PRL-R、EGFP-F/EGFP-R和ACTB-F/ACTB-R为引物(表1)进行PCR扩增, 检测*PRL*和*EGFP*基因的表达情况。提取细胞总蛋白, 用小鼠抗牛PRL体单克隆抗体(Abcam公司)和兔抗β-actin抗体(Cell Signaling公司)分别对转基因细胞总蛋白和未转染的细胞总蛋白进行Western blot分析。

1.5 核移植胚胎的生产

用*Sal* I/*Pvu* I内切酶双切转*PRL*基因载体, 胶回收得到线性化的*PRL*载体。用电转染法将*PRL*转基因载体转入水牛胎儿成纤维细胞系(buffalo fetal fibroblast cells, BFFs), 600 ng/μL G418筛选7 d后, 改用200 ng/μL G418继续筛选7 d, 14 d后荧光显微镜观察, 有绿色荧光的细胞作为核移植供体。对体外成熟的水牛卵母细胞进行去核处理, 供体细胞胰酶消化后显微注射进入已去核的卵母细胞, 电融合后体外培养, 7 d后荧光显微镜观察。

2 结果

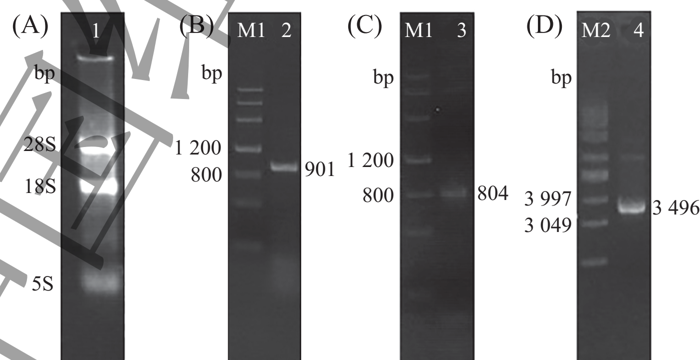
2.1 PRL基因的克隆

采用Trizol试剂法从新鲜成年雌性水牛垂体组织中提取总RNA(图1A)。以总RNA为模板, 经RT-

表1 基因克隆及检测所用引物

Table 1 Primers used in gene cloning and detection

引物名称 Name of primer	引物序列 Sequence of primer	使用目的 Aim
SiPRL-F	5′-AGC CTA GGA CGA GAG CTT C-3′	
PRL-F	5′-CGG GAT CCA TGC ACC ATC ATC ATC ATC ATC TGG TGC CAC GCG GTT CTA CCC CCG TCT GTC CCA AT-3′	Amplificationn of *PRL* gene
PRL-R	5′-GGG GTA CCG ATT TTG ACA TCG CTA CAG AGT-3′	
EGFP-F	5′-ATG GTG AGC AAG GGC GAG GAG-3′	Detection of *EGFP* expression
EGFP-R	5′-TTA CTT GTA CAG CTC GTC CAT G-3′	
ACTB-F	5′-CAC CAC ACC TTC TAC AAT GAG-3′	Detection of *β-actin* expression
ACTB-R	5′-GCG TAC AGG GAT AGC ACA G-3′	



A: 水牛总RNA; B: PCR扩增SiPRL片段; C: PCR扩增*PRL*片段; D: 质粒pPRL。M1: DNA分子量标准III; M2: 超螺旋分子量标准; 1: 总RNA; 2: SiPRL片段; 3: *PRL*片段; 4: 质粒pPRL。

A: the total RNA of water buffalo; B: the SiPRL fragment amplified by PCR; C: the *PRL* fragment amplified by PCR; D: the pPRL vector. M1: DNA marker III; M2: supercoiled DNA ladder marker; 1: the total RNA; 2: SiPRL fragment; 3: *PRL* fragment; 4: pPRL vector.

图1 *PRL*基因克隆

Fig.1 The results of *PRL* gene cloning

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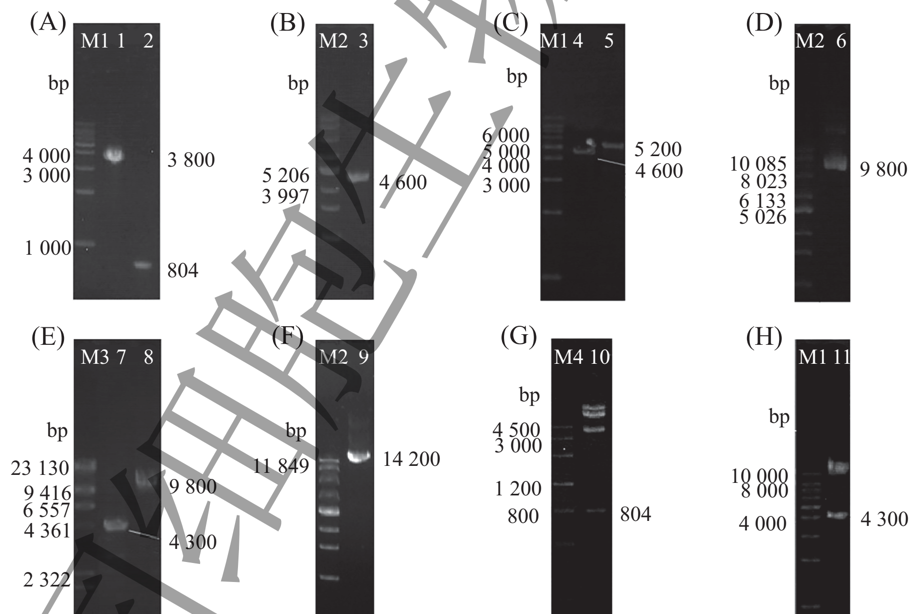
PCR方法扩增得到901 bp含有信号肽的SiPRL片段(图1B), TA克隆得到pSiPRL质粒。以pSiPRL质粒为模板, 扩增得到804 bp不含信号肽的*PRL*片段(图1C), 该片段包含*PRL* mRNA序列中翻译起始位点后91 bp处开始的全部编码框序列, 并在5′端依次加入了*Bam*H I识别位点、翻译起始密码子、6×His蛋白质纯化标签以及凝血酶识位点, 在3′端加入了*Kpn* I识别位点。TA克隆得到大小为3 496 bp的pPRL阳性克隆质粒(图1D)。

2.2 乳腺特异表达*PRL*基因载体的构建

以含有BCNpolyA片段的p-IFN-BCNpolyA载体为骨架, 依次插入*PRL*片段、乳腺特异性*BCN*启动子(娟姗牛β-酪蛋白启动子)、标记基因元件构建乳腺特异表达载体。首先, 用*Bam*H I/*Kpn* I进行双酶切pPRL和pIFN-BCNpolyA质粒, 胶回收得到804 bp的*PRL*和3.8 Kb的p-BCNpolyA片段(图2A), T4连接得到4.6 Kb的pPRL-BCNpolyA克隆(图2B)。接着, 用*Bam*H I/*Sal* I双酶切p5.2BCN和pPRL-BCNpolyA质粒, 分别得到5.2 Kb的BCN和4.6 Kb的pPRL-BCNpolyA片段(图2C), T4连接得到9.8 Kb的p5.2BCN-PRL-BCNpolyA载体(图2D)。然后, 用*Not* I/*Sal* I双酶切p5.2BCN-PRL-BCNpolyA质粒和标记基因pcmv-EGFP-IRES-neo-SV40polyA质粒, 胶回收得到9.8 Kb的p5.2BCN-PRL-BCNpolyA和4.3 Kb的cmv-EGFP-IRES-neo-SV40polyA片段(图2E), T4连接得到大小为14.2 Kb的p5.2BCN-PRL-BCNpolyA-cmv-EGFP-IRES-neo-SV40polyA的核移植用转基因载体(图2F)。最后, 分别用*Bam*H I/*Kpn* I和*Not* I/*Sal* I对该载体进行酶切鉴定, 分别得到与预期均相符的大小为804 bp的PRL片段(图2G)和4.3 Kb的标记基因片段(图2H), 成功构建了乳腺特异性表达*PRL*基因的转基因载体(图3)。



A: 胶回收pBCNpolyA和PRL片段; B: 质粒pPRL-BCNpolyA; C: 胶回收pPRL-BCNpolyA和5.2BCN片段; D: 质粒p5.2BCN-PRL-BCNpolyA; E: 胶回收p5.2BCN-PRL-BCNpolyA和标记基因片段; F: 转基因载体; G: *Bam*H I/*Kpn* I双酶切验证; H: *Not* I/ *Sal* I双酶切验证。M1: 1 Kb DNA分子量标准; M2: 超螺旋DNA分子量标准; M3: λ DNA/*Hin*d III; M4: DNA分子量标准III; 1: p-BCNpolyA片段; 2: *PRL*片段; 3: pPRL-BCNpolyA质粒; 4: pPRL-BCNpolyA片段; 5: 5.2 Kb *BCN*片段; 6: p5.2BCN-PRL-BCNpolyA质粒; 7、11: 标记基因片段; 8: p5.2BCN-PRL-BCNpolyA片段; 9: 转基因载体; 10: *PRL*片段。

A: the p-BCNpolyA and PRL fragments after purification; B: pPRL-BCNpolyA vector; C: the p-BCNpolyA and 5.2BCN fragments after purification; D: the p5.2BCN-PRL-BCNpolyA vector; E: the p5.2BCN-PRL-BCNpolyA and marker gene fragments after purification; F: the transgenic vector; G: the restriction enzyme digestion used *Bam*H I/*Kpn* I; H: the restriction enzyme digestion used *Not* I/*Sal* I. M1: 1 Kb DNA ladder; M2: supercoiled DNA ladder marker; M3: λ DNA/*Hin*d III; M4: DNA marker III; 1: p-BCNpolyA fragment; 2: *PRL* fragment; 3: pPRL-BCNpolyA vector; 4: pPRL-BCNpolyA fragment; 5: 5.2 Kb *BCN* fragment; 6: p5.2BCN-PRL-BCNpolyA vector; 7,11: the marker gene fragment; 8: p5.2BCN-PRL-BCNpolyA fragment; 9: the transgenic vector; 10: *PRL* fragment.

图2 转基因载体的构建

Fig.2 The construction of transgenic vector

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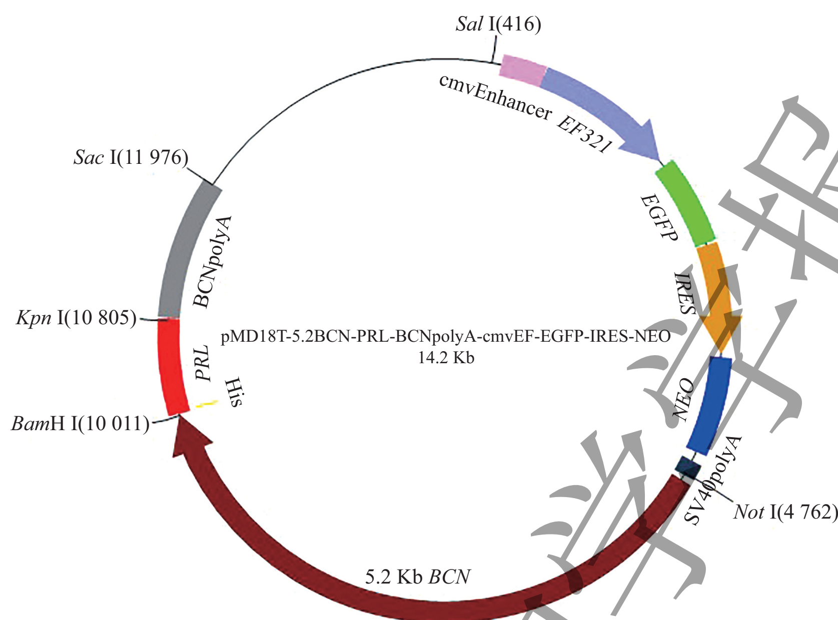
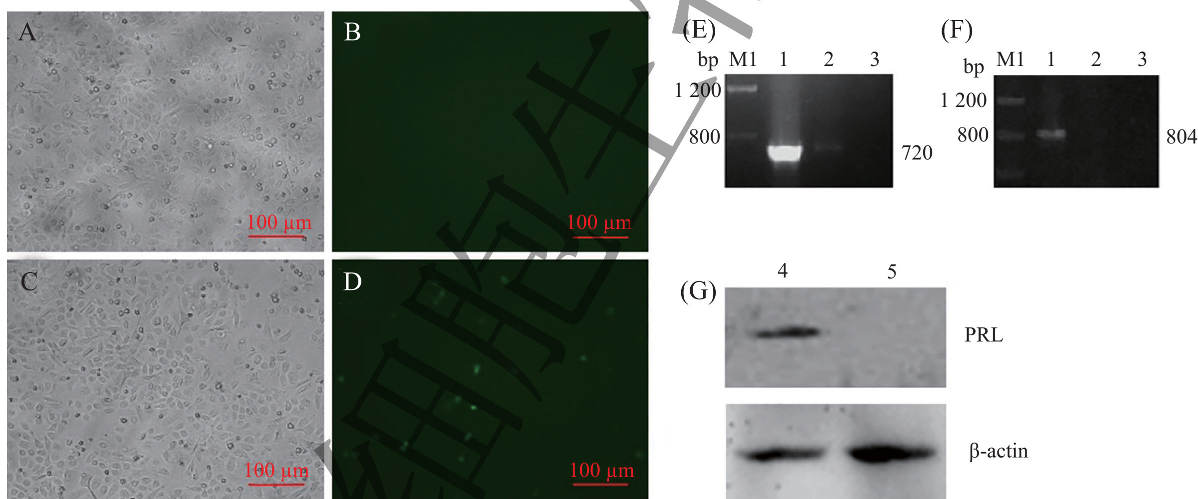


图3 乳腺特异性表达*PRL*基因载体图

Fig.3 The map of mammary gland-specific *PRL* expression vector



A: 常光下的阴性对照组Bcap-37细胞; B: 荧光下的阴性对照组Bcap-37细胞; C: 常光下的转基因组Bcap-37细胞; D: 荧光下的转基因组Bcap-37细胞; E: RT-PCR检测细胞中*EGFP*基因表达情况; F: RT-PCR检测细胞中*PRL*基因表达情况; G: Western blot检测细胞中的PRL蛋白表达情况。M1: DNA分子量标准III; 1: 转基因细胞cDNA; 2: 转基因细胞RNA; 3: 空白对照; 4: 转基因组细胞总蛋白; 5: 阴性对照组细胞总蛋白。

A: the Bcap-37 cells of negative group in the daylight; B: the Bcap-37 cells of negative group in the fluorescent; C: the Bcap-37 cells of transgenic group in the daylight; D: the Bcap-37 cells of transgenic group in the fluorescent; E. the *EGFP* expression in the cells detected by RT-PCR; F: the *PRL* expression in the cells detected by RT-PCR; G: the expression of PRL protein in the cells detected by Western blot analysis. M1: DNA marker III; 1: the cDNA of transgenic cells; 2: the RNA of transgenic cells; 3: the black control; 4: the total protein of transgenic cells; 5: the total protein of negative cells.

图4 转基因载表达情况分析

Fig.4 The expression analysis of transgenic vector

2.3 转*PRL*基因载体转染Bcap-37细胞与检测

用乳腺特异性表达*PRL*基因载体(p5.2BCN-PRL-BCNpolyA-cmv-EGFP-IRES-neo-SV40polyA)和阴性对照(TE buffer)瞬时转染人乳腺癌Bcap-37细胞系。24 h后荧光显微镜观察, 转基因组的Bcap-37细胞有绿色荧光(图4C和图4D), 阴性对照组细胞无荧光(图4A和图4B)。收集转基因组的Bcap-37细胞进行RT-PCR检测, 结果显示, 转基因细胞cDNA可

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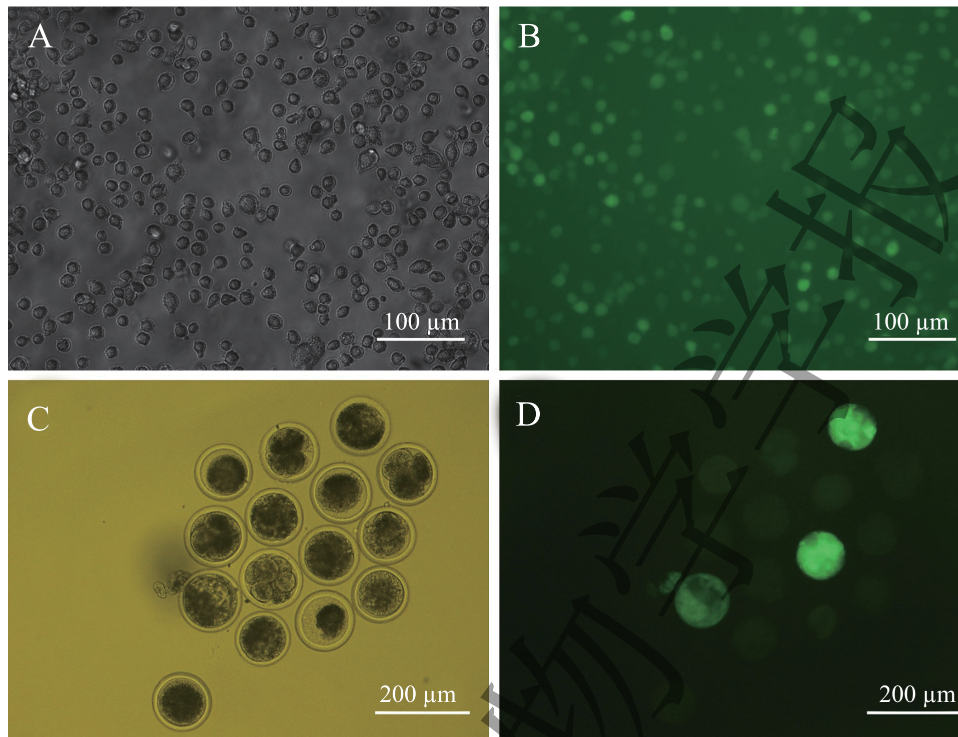

A: 常光下转染*PRL*基因BFFs; B: 荧光下转染*PRL*基因BFFs; C: 常光下转*PRL*基因水牛核移植胚胎; D: 荧光下转*PRL*基因水牛核移植胚胎。
A: the *PRL* transgenic BFFs in the daylight; B: the *PRL* transgenic BFFs in the fluorescent; C: the *PRL* transgenic cloning buffaloembryos in the daylight produced by nuclear transfer; D: the *PRL* transgenic cloning buffaloembryos in the fluorescent produced by nuclear transfer.

图5 转*PRL*基因水牛克隆胚胎

Fig.5 The *PRL* transgenic cloning buffalo embryos

扩增得到720 bp的*EGFP*基因条带(图4E)和804 bp的*PRL*基因条带(图4F), 而未经反转录的RNA样本以及空白对照均无扩增条带。对2组细胞进行Western blot分析发现, 转基因组的细胞有外源PRL蛋白表达, 阴性对照组不表达(图4G)。以上结果说明, 构建的乳腺特异性转基因载体可在Bcap-37细胞系中表达外源*PRL*基因。

2.4 转*PRL*基因水牛克隆胚胎的生产

用电转染法将*PRL*转基因载体转入水牛胎儿成纤维细胞系(BFFs), 经新霉素筛选获得转*PRL*基因BFFs, 胰蛋白酶消化后作为核移植用细胞, 荧光显微镜下可见绿色荧光(图5A和图5B)。将该细胞注入已去核的水牛卵母细胞, 经体外培养获得转*PRL*基因水牛核移植克隆胚胎, 荧光显微镜下可见绿色荧光(图5C和图5D), 表明利用该载体可产出转*PRL*基因水牛克隆胚胎。

3 讨论

常见的乳腺特异性启动子有乳清蛋白和酪蛋白启动子。一些研究发现, 乳清蛋白启动子的组织特异性较弱。在*WAP*(whey acidic protein)启动子表达*hGH*(human growth hormone)基因小鼠模型中, 外源*hGH*基因在公母小鼠的非乳腺组织中均有表达[22]。与乳清蛋白相比, 酪蛋白启动子的组织特异性较强。在山羊αS1-酪蛋白启动子表达*hG-CS*(human granulocyte colony-stimulating factor)基因小鼠中, 未发现外源基因在乳腺外表达[23]。娟姗牛β-酪蛋白启动子在表达人干扰素α2b小鼠模型中, 未发现外源基因在乳腺外表达[24-25]。因此, 酪蛋白启动子作为乳腺特异性启动子得到了越来越多的应用。李辉等[26]利用*BCN*启动子在Bcap-37细胞系中成功表达具有生物活性的人干扰素α2b。本文利用该启动子构建的转*PRL*基因载体也能在Bcap-37细胞系中表达目的*PRL*基因。

在传统的转基因载体构建中, 一个转基因载体通常仅含一个表达单元, 目的基因与标记基因间用IRES(internal ribosome entry site)或2A间隔, 保证两个蛋白共同转录并分开翻译, 以通过标记基因跟

踪目的基因的表达情况。本文中启动目的基因表达的*BCN*启动子为乳腺特异性启动子, 若以该启动子调控标记基因, 则标记基因无法在非乳腺细胞系和转基因动物胚胎中表达, 不能发挥标记作用。为此, 本文构建的转基因载体含顺势串联的2个表达单元——乳腺特异性*BCN*启动子(目的基因*PRL*)和非组织特异性cmv(cytomegalovirus)启动子(标记基因), 标记基因的表达虽然不能直接表明目的基因表达与否, 但能指示是否为转基因细胞或胚胎。且本课题组前期研究结果表明, 在转基因载体构建中, 采用IRES启动外源药物抗性基因的表达更有利于转基因阳性细胞的筛选(结果未发表)。本研究亦发现, 采用IRES-Neo连接方式, 通过该*PRL*表达载体转染水牛胎儿成纤维可筛选出高阳性率的转基因细胞系, 以该细胞系为供体细胞, 通过核移植法可生产出转*PRL*基因水牛克隆胚胎。

本研究克隆了水牛*PRL*基因编码序列, 并以乳腺特异性*BCN*为目的基因*PRL*的启动子, 采用乳腺特异性的*PRL*基因表达单元和非组织特异性的标记基因表达单元相串联的结构, 构建了乳腺特异性表达*PRL*基因的核移植载体。该载体可在乳腺细胞系中表达目的基因, 并通过核移植法获得了转*PRL*基因水牛胚胎。本文首次构建了适用于核移植法生产转*PRL*基因的载体, 并通过核移植法验证了该载体的有效性, 为通过分子育种途径提高水牛产奶量或奶品质奠定了基础。但外源表达*PRL*基因对活体奶水牛泌乳量、奶品质以及繁殖等方面的影响还有待今后进一步研究。

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